MINIREVIEW—MOLECULAR PHARMACOLOGY IN CHINA

Advances in Hypoxia-Mediated Mechanisms in Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most common and the third most deadly malignant tumor worldwide. Hypoxia and related oxidative stress are heavily involved in the process of HCC development and its therapies. However, direct and accurate measurement of oxygen concentration and evaluation of hypoxic effects in HCC prove difficult. Moreover, the hypoxia-mediated mechanisms in HCC remain elusive. Here, we summarize recent major evidence of hypoxia in HCC lesions shown by measuring partial pressure of oxygen (pO_2), the clinical importance of hypoxic markers in HCC,

and recent advances in hypoxia-related mechanisms and therapies in HCC. For the mechanisms, we focus mainly on the roles of oxygen-sensing proteins (i.e., hypoxia-inducible factor and neuroglobin) and hypoxia-induced signaling proteins (e.g., matrix metalloproteinases, high mobility group box 1, Beclin 1, glucose metabolism enzymes, and vascular endothelial growth factor). With respect to therapies, we discuss mainly YQ23, sorafenib, 2-methoxyestradiol, and celastrol. This review focuses primarily on the results of clinical and animal studies.

Introduction

Liver cancer is presently the fifth most prevalent malignant tumor and the third leading cause of cancer-related death worldwide. Primary hepatocellular carcinoma (HCC) or malignant hepatoma accounts around 80% of liver cancers. In the U.S., it was estimated that there were approximately 33,190 newly diagnosed liver cancer cases and 23,000 liver cancer deaths in the year 2014, representing a steady rise in liver cancer incidence (1975–2010) and mortality (1992–2010) (Siegel et al., 2014). In China, the incidence and mortality of liver cancers have increased rapidly and the number of liver cancer deaths was ranked in the top two among all types of cancers in 2014 (McGuire, 2016). The high mortality of liver cancers reflects the general ineffectiveness of current HCC therapies.

Epidemiologically, HCC occurs mainly in Asia, sub-Saharan Africa, North America, and Western Europe and is correlated with the prevalence of hepatitis B virus (in China) or hepatitis

C virus (in Japan), or with infection and alcoholism (in Western Europe). Aflatoxin and liver cirrhosis are also important risk factors for HCC (Zhang et al., 2015). The development of HCC is a complicated pathologic process in which hypoxia and related oxidative stress are common pathophysiological factors associated with infection/inflammation or cellular toxicity/injury after exposure to various risk factors (Severi et al., 2010). Chronic hypoxia and related oxidative stress induce profound epigenetic/genetic alterations in hepatocytes, accompanying repetitive-injury regeneration of hepatocytes upon risk factor exposure. These responses lead to cellular stress adaptation and ultimately to HCC carcinogenesis (Nishida and Kudo, 2013).

HCC is treated mainly by surgical resection, liver transplantation, chemotherapy (e.g., sorafenib), interventional chemotherapy [e.g., transcatheter arterial chemoembolization (TACE)], and/or radiation. Hypoxia or ischemia always accompany and then follow HCC therapies and heavily affect the therapeutic outcomes. In HCC interventional therapies, ischemic death is a major therapeutic mechanism. In addition, hypoxic responses to therapy have important prognostic value for HCC (Hao et al., 2013; Hayano et al., 2014; Ippolito et al., 2014).

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ABBREVIATIONS: 2-ME2, 2-Methoxyestradiol; CA-IX, carbonic anhydrase IX; CT, computerized tomography; Cygb, cytoglobin, DFS, disease-free survival; EGFR, epidermal growth factor receptor; GLUT1, glucose transporter 1; H2AX, H2A histone family, member X; γ -H2AX, phosphorylated H2AX; HCC, hepatocellular carcinoma; HIF-1 α /2 α , hypoxia inducible factor-1 α /2 α ; HMGB1, high-mobility group box 1 protein; IL, interleukin; miR, microRNA; LSD1, lysine-specific demethylase-1; MMPs, matrix metalloproteinases; mtDNA, mitochondrial DNA; NF, nuclear factor; Ngb, neuroglobin; OS, overall survival; PFS, progression-free survival; PK, pyruvate kinase; PTEN, phosphatase and tensin homolog; SDF1, stromal cell-derived factor-1; STAT, signal transducer and activator of transcription; TACE, transcatheter arterial chemoembolization; TIMP, tissue inhibitor of metalloproteinases; TLR, Toll-like receptor; TNM, tumor-node-metastasis; VEGF, vascular endothelial growth factor.

Hypoxia is considered to be involved in HCC development and therapy; however, the exact roles hypoxia plays remain elusive. The major reasons include the following: 1) direct measurement of partial pressure of oxygen (pO_2) in patient HCC lesions is rare; 2) there is no specific signaling molecule or receptor for hypoxia; and 3) hypoxic responses are extremely complicated. In this review, we first discuss the direct evidence and clinical significance of hypoxia in HCC lesions. We then summarize most recent advances in hypoxia-related mechanisms and therapies in HCC.

Direct Evidence of Hypoxia in HCC Lesions by Measuring pO_2

Pathophysiologically, hypoxia refers to insufficient oxygen supply to a cell/tissue/organ or impaired cellular oxygen utilization that finally results in malfunction of the cell/tissue/organ. The liver accepts both oxygenated blood from the hepatic artery (~30%) and deoxygenated blood from the portal vein (~70%). The oxygen tensions of the influent and effluent blood in the hepatic sinusoid (supplying oxygen to hepatocytes) are 60–65 and 30–35 mmHg, respectively, lower than that of most other tissue capillaries (74–104 and 34–46 mmHg) (Jungermann and Kietzmann, 2000). Further, blood flow in the hepatic sinusoid is relatively slow, whereas the metabolic rate of hepatocytes is relatively high. Therefore, hepatocytes are particularly vulnerable to primary or secondary hypoxia after viral infection, toxic substances exposure, or inflammation. Li et al. (2015) reported that the liver is one of the three organs most vulnerable to hypoxia, as determined by exposing rats to high-altitude anoxia (pO_2 20 mmHg) compared with sea level pO_2 (83 mmHg). Upon exposure to endotoxin, mouse liver sinusoidal pO_2 was reduced by 75% from 5 to 44 mmHg (15 minutes) to 11 mmHg (6 hours), although cardiac output, arterial oxygen saturation, and blood flow in the hepatic artery were unaffected (James et al., 2002). In patients with liver cirrhosis, hypoxemia ranges from 10 to 40% depending on hepatic dysfunction (Møller et al., 1998). Hypoxia profoundly interferes with immune responses and cell survival/death machinery and induces epigenetic/proteomic/genomic alterations as a result of the reduced pO_2 level associated with cell malignancy (Höckel and Vaupel, 2001). After exposing mouse tumor cells to pO_2 of <1 mmHg for 4 hours, the mutation rate was increased by 3.4-fold compared with the normoxic control (Reynolds et al., 1996). Since the liver is frequently exposed to various toxic insults, and hypoxia can be easily induced in the liver after injury, it is conceivable that repetitive or chronic hypoxia occurs during pathologic situations such as liver cirrhosis. Considering the prominent effect of hypoxia in inducing gene mutation, repetitive or chronic hypoxia might be a driving force for carcinogenesis of hepatocytes.

During the process of tumor development, cancer cells inside a solid tumor can suffer from three major types of hypoxia, chronic diffusion hypoxia, acute/intermittent perfusion hypoxia, or/and anemic hypoxia, owing to the abnormal growth of tumor vasculature, increased tumor size, and reduced oxygen concentration in the blood. A precise diagnosis of intratumor hypoxia is difficult in patients, particularly at the cellular level. Hypoxia is associated with many factors, including peripheral pO_2 , oxygen saturation/content in capillaries, tissue blood perfusion volume, the distance between

cells and capillaries, amount and quality of cellular mitochondria, and oxygen demand of cells. Among all hypoxic parameters, intratumor pO_2 is the most direct indicator of tumor hypoxia (Höckel and Vaupel, 2001).

At the initial stage of development of a solid tumor, cancer cell proliferation overwhelms tumor angiogenesis. Therefore, cancer cells approximately 70 μ m away from the oxygenated blood may suffer from diffusion hypoxia since oxygen diffusion decreases within 100–200 μ m of a functional capillary. Liu et al. (2014) examined tumor oxygenation by using OxyLab pO_2 in an orthotopic rat HCC model and found that most regions inside the tumor (74.1%) had pO_2 values of 0–10 mmHg. When pO_2 was measured using a fluorescence fiberoptic oxygen probe, it was found that the pO_2 inside tumors ranged from 0.2 to 0.8 mmHg in three rat liver tumors with diameters of 0.7, 1.0, and 1.5 cm; this pO_2 value was significantly lower than that in normal liver tissue (45 mmHg) (Riedl et al., 2008). These findings are consistent with the fact that intratumor pO_2 is severely decreased in other types of solid cancers (e.g., primary uterine cervix cancers) at their early stages (Vaupel et al., 2007). Direct intratumor polarographic measurement of pO_2 is the “gold standard” method for measuring intratumor hypoxia. In four patients with liver metastases of rectal cancers, the measured median pO_2 inside tumors was 6 mmHg, much lower than that in normal liver tissues (30 mmHg) (Vaupel et al., 2007). Polarography data measuring pO_2 in primary HCC are rare.

During development of a tumor, tumor angiogenesis is prominent and is induced mainly by hypoxia-induced vascular endothelial growth factor (VEGF) produced in cancer cells. The vasculature in HCC is reconstructed profoundly from dominant portal perfusion to dominant hepatic arterial perfusion, causing larger HCC to become perfusion-rich tumors (Chou et al., 2014). Taouli et al. (2013) reported an increase in the arterial blood flow/fraction and a decrease in portal venous blood flow in HCC (tumor size: 1.1–12.6 cm, $n = 26$), compared with liver parenchyma, by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). Similarly, Chen et al. (2016) reported that advanced HCC had significantly higher peak (maximal perfusion signal intensity), slope (maximal ascending slope of perfusion curve), arterial fraction (hepatic artery/portal vein perfusion), and arterial flow, but lower portal flow, distribution volume, and mean transit time compared with surrounding liver parenchyma ($n = 92$) as measured by DCE-MRI. The overall survival (OS) of patients with higher peak blood flow in their tumors is significantly improved, suggesting that hypoxia is an adverse factor for HCC patient survival. Similar results were also reported from studies using perfusion computerized tomography (CT) detection (Arizumi et al., 2014; Bayraktutan et al., 2014; Guo and Yu, 2014). Owing to the abnormal vessel structure or function, acute/intermittent perfusion hypoxia is common in HCC. It is generally believed that heterogeneous microregional hypoxia is widely distributed inside HCC, as has been shown in other types of cancers (Vaupel et al., 2007). Guo and Yu (2014) examined the perfusion of tumor tissue in 45 patients with HCC by four-dimensional (4D) CT and found that a cancer-feeding artery was present in only 28 cases. Among these 28 cases, 20 had thickened, rigid, or distorted feeding arteries. Moreover, 14 cases had a thrombus in the portal veins (Guo and Yu, 2014). In another study, regional tumor

blood flow (20.6–105.7 ml/100 g per minute) and oxygen extraction fraction (20.4–56.7%)/oxygen metabolic rate (1.71–5.05 ml/100 g per minute) varied greatly between individual HCC ($n = 6$) as measured by perfusion CT (Fukuda et al., 2004). Together, these studies suggest malfunction of arterial perfusion inside HCC despite the increased arterial perfusion volume. Secondary hypoxia in HCC after treatments such as TACE is common and is an important indicator of therapeutic effectiveness and an independent predictor for HCC prognosis (Hao et al., 2013; Hayano et al., 2014; Ippolito et al., 2014); this topic is beyond the focus of this review. Although measurements of perfusion parameters can indicate tumor hypoxia, measuring pO_2 in primary HCC using an intratumor polarographic oxygen-sensor is indispensable for diagnosing tumor hypoxia in HCC and will be an important parameter for precise targeting of hypoxia in HCC.

Clinical Importance of Hypoxic Markers in HCC

Clinically, hypoxia is considered as an independent adverse prognostic factor for HCC. Lai et al. (2015) reported that carbonic anhydrase IX (CA-IX, a hypoxia marker) was expressed in 19 of 40 (47.5%) residual/recurrent HCCs (after TACE) and 2 of 17 (11.8%) untreated HCCs. Huang et al. (2015) reported that CA-IX was detected in 110 of the 227 (48.5%) unifocal and respectable primary HCC tumors, correlating with younger age, female sex, larger tumor size, higher tumor grade and higher tumor stage. CA-IX-positive HCC patients had a lower 5-year overall survival/disease-free survival (OS/DFS). Furthermore, CA-IX was a poor predictor of DFS and OS in advanced HCC. After analyzing the relationship between hypoxia markers (HIF-1 α and CA-IX) and patient survival parameters (i.e., OS and recurrence-free survival) in 179 primary HCC, Srivastava et al. (2015) demonstrated that high expression levels of HIF-1 α and CA-IX appeared in 60% and 15% of cases, respectively, correlating with a worse prognosis independent of antigen Ki-67 expression. When hypoxia markers and Wnt pathway markers were coexpressed, the OS in HCC (tumor size <5 cm) was worse. Similar results regarding the role of HIF-1 α in HCC prognosis were reported by Osman et al. (2015). In this study, HIF-1 α was overexpressed in 42 of 65 HCC specimens (64.6%), correlating to larger tumor sizes, more tumor loci, and more advanced stages of the disease. Further, the authors found that the expression of autophagy marker Beclin-1 was associated with that of HIF-1 α in HCC. In the high HIF-1 α group, but not low HIF-1 α group, more HCC cells were Beclin-1-positive, suggesting that hypoxia might activate autophagy during HCC development. The clinical significance of Beclin-1 expression in HCC will be discussed in the following section. In addition to HCC specimens, HIF-1 α levels were also increased in the serum of patients with HCC compared with healthy populations and with patients with liver cirrhosis, and showed a significant correlation with nuclear factor (NF)- κ B p65 expression/activity and also poor prognosis of HCC (Gaballah et al., 2014). Wang et al. (2014) reported that HIF-1 α mRNA levels ($n = 32$) and protein levels ($n = 33$) were upregulated in HCC lesions compared with adjacent nontumor tissues, correlating with larger tumor sizes, metastasis, advanced disease stage, and shorter survival time. In HCC after liver transplantation, higher HIF-1 α levels were

also significantly associated with tumor invasion, advanced TNM stages, as well as shorter OS in 31 patients (Xiao et al., 2014). The authors compared the effect of preoperative TACE with that of non-TACE on HCC prognosis ($n = 25$) and found that in TACE-treated HCC ($n = 10$) an increase in HIF-1 α expression was correlated to an increase in the 2-year recurrence rate and shorter disease-free survival (Xu et al., 2014). However, a meta-analysis of 851 HCC cases from eight studies by Cao et al. (2014) showed that HIF-1 α protein levels were correlated only with vascular invasion or worse DFS but not to other HCC pathologic characteristics (e.g., capsule formation, cirrhosis, tumor size, and tumor differentiation). Together, the results of these studies support HIF-1 α as a reliable indicator of prognosis for all HCC, whereas CA-IX is still not reliable because its expression in HCC cases varies greatly (11.8–48.5%) between different studies.

Hypoxia-Related Mechanisms in HCC

It is generally believed that hypoxia plays important roles in hepatocarcinogenesis, HCC development, and HCC recurrence after chemotherapy by promoting HCC cell proliferation/invasion and angiogenesis. HIF-1 α is the most commonly investigated player in HCC hypoxic responses. HIF-2 α , matrix metalloproteinases (MMPs), high mobility group box 1 (HMGB1), Beclin 1, and glucose metabolism enzymes are recently investigated molecules that are also involved in hypoxia-induced effects in HCC. In addition, neuroglobin (Ngb) is a novel intracellular O_2 -binding protein that may directly sense pO_2 changes in HCC cells. These molecules might collaborate to sense and transduce hypoxic signaling in HCC cells during HCC development and therapies (Fig. 1, Table 1).

Role of HIF-1 α in HCC

HIFs (including HIFs 1, 2, and 3) are heterodimeric transcription factors (α and β subunits) that are responsive to reduced cellular oxygen supply. In the presence of oxygen, HIF prolyl-hydroxylase catalyzes the hydroxylation of HIF-1 α at proline residues, leading to rapid proteasomal degradation of HIF-1 α via a von Hippel-Lindau protein-dependent mechanism. During hypoxia, the activity of HIF prolyl-hydroxylase is inhibited. This allows HIF-1 α to accumulate and bind to HIF-1 β , forming a stabilized HIF-1 α /1 β dimer. The dimer enters into the nuclei to exert its transcriptional activity by binding to the HIF-responsive elements in promoters (containing NCGTG sequence) of a variety of genes, including glycolysis enzymes and VEGF.

HIF-1 and NF- κ B are inter-regulated transcriptional factors during hypoxia and inflammation. Most studies demonstrate that HIF-1 enhances NF- κ B transcription mainly during acute hypoxia, whereas NF- κ B promotes the expression of HIF-1 α under normal conditions and in response to inflammatory stimuli [e.g., tumor necrosis factor α and oxygen reactive species (ROS)]. Recently, Jiang et al. (2015) demonstrated that only the p50/p65 subunits of NF- κ B upregulated HIF-1 α upon acute hypoxia, whereas the c-Rel subunit of NF- κ B downregulated HIF-1 α during prolonged hypoxia via miR-93 and miR-199a-5p in HCC cells. Meanwhile, HIF-1 α upregulated Dicer1 [a key enzyme in mature micro-RNA (miR)

Fig. 1. Hypoxia-related mechanisms in HCC. Hypoxia upregulates/activates HIF-1 α , HIF-2 α , γ -H2AX, PIM1, HMGB1, Rab11-FIP4, PAI-1, SERPINB3, IGFBP1, MMPs, and TIMP2 either directly or indirectly, which further regulates downstream signaling proteins to promote or suppress HCC cell proliferation, migration, and invasion. In addition, PKM2, EGFR, miRs, TLR4/9, and autophagy play roles in the regulation of hypoxia-mediated signaling pathways. Independently, neuroglobin (Ngb) senses hypoxia signal via binding to O₂ and by regulating Raf-1, HIF-1 α , Gai, AKT, PTEN, and 14-3-3 proteins simultaneously. The binding of Ngb to Raf-1 leads to inactivation of Raf-1-MEK-Erk1/2 pathway and prevents HCC cell proliferation/migration. $\rightarrow$, upregulation/activation; $\perp$, Downregulation/inhibition; 14-3-3, 14-3-3 proteins; AKT, AKT serine/threonine kinase 1; c-Rel, reticuloendotheliosis oncogene C; Dicer 1, dicer 1, ribonuclease III; EGF, epidermal growth factor; Erk1/2, extracellular signal-regulated kinase 1/2; Gai, G-protein alpha subunit; IGFBP1, insulin-like growth factor binding protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; P60/p65, protein of 60/65 kDa; PAI-1, plasminogen activator inhibitor 1; PIM1, serine/threonine-protein kinase pim-1; PKM2, pyruvate kinase 2, muscle; PRA S40, proline-rich AKT substrate of 40 kDa or AKT1 substrate 1; PTEN, phosphatase and tensin homolog; Rab11-FIP4, Rab11-family interacting protein 4; SERPINB3, serpin peptidase inhibitor 3; STAT3, signal transducer and activator of transcription 3.

generation] and fine-tuned HIF-1 α levels in HCC cells during hypoxia via a miR-mediated negative-feedback mechanism. In addition, p65 can positively regulate HIF-1 α expression in HCC cells during hypoxia by interacting with signal transducer and activator of transcription 3 (STAT3) (Won et al., 2015).

Pyruvate kinase (PK) is involved in glycolysis and limits the rate of aerobic glycolysis. Human PK genes (L, R, M1, and M2 isoforms) include PKLR and PKM (Mazurek, 2011). It is known that PKM2 is overexpressed in many cancer cell lines, where it promotes aerobic glycolysis. Recently, Dong et al. (2015) demonstrated that PKM2 was overexpressed in HCC

TABLE 1
Hypoxia-targeted genes and their functions in HCC cells

Hypoxic Treatment	Hypoxic Targets	Hypoxia-Targeted Gene Functions	Cell Lines, Animal Models, or Human Samples	Reference
1% O ₂ 4 hr	p50/p65↑, HIF-1α↑, c-Rel↑, Dicer1↓	NF-κB p50/p65/c-Rel binds the HIF-1α promoter and increases its transcription, whereas Dicer1 is downregulated in acute hypoxia.	HepG2, Huh7	Jiang et al., 2015
1% O ₂ 24 hr	Dicer 1↓, miR-199a-5p/93↑, HIF-1α↓	Upregulation of Dicer/c-Rel downstream miRs suppress HIF-1α expression in prolonged hypoxia.	HepG2, Huh7	Jiang et al., 2015
1% O ₂ 6–40 hr	p-STAT1, CD133↑	STAT3/NF-κB induces CD133 expression via IL-6/STAT3/HIF-1α; CD133 induces HCC tumor growth in vivo and is co-upregulated with HIF-1α/STAT3 in hHCC.	Huh7, PLC/PRF/5, SNU-398, Hep3B, HepG2 and SNU-449, Balb/c nude mice/Huh7, hHCC tissues,	Won et al., 2015
1% O ₂ 2–24 hr	γ-H2AX↑	γ-H2AX upregulates VEGF via COX2/EGFR/HIF-1α signaling.	HepG2, BEL-7402	Xiao et al., 2015
1% O ₂ 6–48 hr or CoCl ₂ 24 hr	HIF-1α↑, Rab11-FIP4↑	Hypoxia induces Rab11-FIP4 expression via HIF-1α; Rab11-FIP4-p-PRAS40 promotes HCC cell migration, invasion and metastasis in vitro and in vivo.	SK-Hep1, MHCC97H, HCCLM3, HepG2, Huh7, SK-Hep1, Balb/c nude mice/HCCLM3	Hu et al., 2015
1% O ₂ 16 hr + disulfiram	HIF-2α↓	Disulfiram downregulates HIF-2α-EPO/CA9/PDK1-VEGF.	Hep3B, Huh7, HepG2	Park et al., 2013
1% O ₂ 48 hr + Sorafenib	HIF-2α↑	HIF-2α contributes to hypoxia-induced sorafenib-resistance in HCC cells via enhancing TGF-α/EGFR signaling.	HepG2, Huh7, nude mice/Huh7	Zhao et al., 2014
1% O ₂ 4 hr+ HIF-2α KD	PAL-1↓	HIF-2α suppresses CD31 via PAI/plasmin signaling.	HepG2+CGR8 tumor spheroid-embryonic body-derived cocultures	Geis et al., 2015a
0.1–3% O ₂ 1–96 hr	HIF-2α↑, SERPINB3↑	HIF-2α enhances SERPINB3 via binding to its promoter in hypoxic HCC cells; HIF-2α and SERPINB3 are co-upregulated in hHCC.	HepG2, Huh7, HT-29, hHCC tissues	Cannito et al., 2015
1% O ₂ 16 hr + HIF-2α KD	IGFBP1↓	HIF-2α KD increases VEGFR3/LYVE-1 via IGFBP1/IGF signaling.	HepG2+CGR8 tumor spheroid-embryonic body-derived co-cultures	Geis et al., 2015b
1% O ₂ 48 hr + SDF-1	MMP10↑	SDF-1 upregulates MMP10 via CXCR4/MEK signaling.	Huh7	García-Irigoyen et al., 2015
0.1% O ₂ 48 hr	TIMP2↓	TIMP2 KD enhances cell invasion via HIF-1α/miR-210/HIF-3α regulatory feedback circuit.	SMMC-7721, PLC/PRF/5, MHCC-97L, BEL-7402	Kai et al., 2016
1% O ₂ 24 hr	TLR9↑, HMGB1↑	TLR9 interacts with HMGB1 in HCC cells and promotes HCC development in vivo; TLR9 and HIF-1α are co-upregulated in hHCC.	Hepa1-6, Huh7, C57 mice/Hepa1-6, hHCC tissues	Liu et al., 2015
1% O ₂ or CoCl ₂ 12 hr + LSD1 KD	HIF-1α↓	LSD1 KD reduces glycolytic activity via suppression of HIF-1α/GLUT1 signaling but activates mitochondrial respiration via enhancing H3K4 methylation.	HepG2, Huh7, SCID mice/HepG2	Sakamoto et al., 2015
1% O ₂ 6 hr + PIM1 KD	EMT1, p-AKT1, GLUT1↓	PIM1 KD suppresses HCC cell proliferation, invasion, and EMT and dampens glycolysis via decreasing p-AKT, PKM2 and GLUT1	SMMC-7721, SMMC-97L, Balb/c nude mice/SMMC-7721	Leung et al., 2015

COX2, cyclo-oxygenase 2; EMT, epithelial-mesenchymal transition; GLUT1, glucose transporter 1; hHCC, human hepatocellular carcinoma; KD, knockdown; LSD1, lysine-specific demethylase 1; TFPDP3, transcription factor dimerization partner 3; TGF, tumor growth factor.
↑, upregulation/activation; ↓, downregulation/inactivation.

samples. Further, these authors showed that PKM2 can phosphorylate STAT3 at tyrosine 705, which further upregulates HIF-1 α and promotes HCC cell proliferation. This evidence suggests a molecular link between glucose metabolism and hypoxic responses involving PKM2-STAT3-HIF-1 α signaling. Phosphorylated H2A histone family, member X (γ -H2AX) is a common indicator of DNA damage/repair in HCC (Liu et al., 2012a; Matsuda et al., 2013). Xiao et al. (2015) demonstrated that γ -H2AX was induced in HCC cells upon hypoxia. Further, the increased γ -H2AX expression correlated to larger HCC tumor sizes, advanced TNM stages, and poor OS in HCC after liver transplantation. Knockdown γ -H2AX effectively suppressed mRNA levels for cyclooxygenase 2 and epidermal growth factor receptor (EGFR) in BEL-7402 and HepG2 cells, and partially suppressed HIF-1 α and VEGF expression upon hypoxia. Further, knockdown of EGFR effectively downregulated HIF-1 α and VEGF in hypoxic HCC cells. These results suggest that γ -H2AX regulates hypoxic responses in HCC cells via an EGFR-HIF-1 α -VEGF axis. Clinically, higher γ -H2AX expression combined with HIF-1 α and EGFR provided a more valuable predictor for patients with poor HCC prognosis after liver transplantation.

Rab11-FIP4 (Rab11-family interacting protein) probably functions as a downstream target of HIF-1 α in hypoxic HCC cells. In HCC samples, Rab11-FIP4 expression was positively related to HIF-1 α expression (Hu et al., 2015). Clinical data revealed that HIF-1 α combined with Rab11-FIP4 indicates poor prognosis more reliably. Mechanistically, Rab11-FIP4 promoted PRAS40 (proline-rich Akt substrate) expression, HCC cell mobility/invasion, and remote lung metastasis of HCC cells in mice. Considering that PRAS40 is a key downstream target of the mTOR, HIF-1 α may play a role in regulating autophagy via the Rab11-FIP4/mTOR-PRAS40 pathway in HCC cells in response to hypoxia.

Role of HIF-2 α in HCC

Compared with HIF-1 α , HIF-2 α has been less studied in HCC. A recent meta-analysis of 1066 Chinese HCC patients from seven independent studies showed that higher HIF-2 α correlated well to the increased capsule infiltration/vein invasion/histologic grade but not with HCC prognosis (Yao et al., 2015). HIF-2 α and HIF-1 α were prominently upregulated in HCC cells upon 16 hours of hypoxic (1% O₂) incubation (Park et al., 2013). Knockdown of HIF-2 α enhanced autophagic activity, attenuated apoptosis (Menrad et al., 2010), and enhanced the effects of doxorubicin (He et al., 2012) or sorafenib (Zhao et al., 2014) on suppressing the development of HCC xenografts in mice. These findings suggest an oncogenic effect of HIF-2 α in HCC cells. However, there are also studies (Sun et al., 2013; Yang et al., 2016) reporting that overexpression of HIF-2 α induced higher levels of apoptosis and inhibited HCC tumor growth in mice, suggesting a tumor suppressor function for HIF-2 α in HCC. Yang et al. (2016) reported that HIF-2 α was decreased in HCC lesions compared with peritumoral tissues ($n = 206$) and that patients with high HIF-2 α had longer OS. A previous study (Menrad et al., 2010) showed that knockdown of HIF-1 α increased HIF-2 α and that knockdown of HIF-2 α increased HIF-1 α in HCC cells, suggesting a balance between HIF-1 α and HIF-2 α in HCC cells. Sun et al. (2013) reported that transcription factor dimerization partner 3 (TFDP3) was a downstream target of HIF-2 α in HCC

cells. Knockdown of HIF-2 α downregulated the expression of VEGF/cyclin D1/tumor growth factor α and inhibited EGFR activation (Zhao et al., 2014). Geis et al. (2015a) identified plasminogen activator inhibitor 1 (PAI-1) as an HIF-2 α targeted gene in HepG2 cells by microarray assay. Cannito et al. (2015) demonstrated that HIF-2 α but not HIF-1 α bound to the promoter of SERPINB3 gene (a cysteine-proteases inhibitor) and upregulated SERPINB3 gene expression in hypoxic HCC cells. In addition, insulin-like growth factor binding protein 1 (IGFBP1) was identified as an HIF-2 α targeted gene that links HIF-2 α to insulin-like growth factor (IGF) signaling (Geis et al., 2015b). Taken together, HIF-2 α plays diverse roles in regulating hypoxic responses of HCC cells, distinct from those of HIF-1 α , in certain cellular contexts.

Role of MMPs in HCC

MMPs are key molecular players in regulating tumor microenvironment (Kessenbrock et al., 2010). Several members of the MMP family (including MMPs 1, 2, 3, and 9) were upregulated in human HCC tissues (Okazaki and Inagaki, 2012), which contributed to the migration/invasion of HCC in vitro (Chen et al., 2013). Recently, MMP-8 and MMP-10 were also found to be highly associated with human HCC. Qin et al. (2016) reported that MMP-8 was coexpressed with tumor growth factor β 1 in highly aggressive-HCC patients. García-Irigoyen et al. (2015) reported that MMP10 was upregulated in both human and diethylnitrosamine-induced mouse hepatoma. In the MMP10-knockout mice treated with diethylnitrosamine, not only was the incidence of HCC decreased, but the tumor size, vascularization, and lung metastasis were also reduced. During hypoxia, MMP10 was upregulated in HCC cells via Erk-mediated signaling pathways (García-Irigoyen et al., 2015). In addition, MMP10 could be upregulated by carboxylic acid-truncated hepatitis B virus X protein (HBx Δ C1) in HCC cells (Sze et al., 2013). Mutation of HBx Δ C1 at its binding sites for the MMP10 promoter abolished the effect of HBx Δ C1 on MMP10 induction. Silencing MMP10 in HBx Δ C1-expressing HepG2 cells inhibited cell migration/invasion. Since HBx Δ C1 is expressed in 46% of HCC and is highly associated with tumor invasion, HBx Δ C1-MMP10 signaling pathway may be crucial for HCC progression.

Tissue inhibitor of metalloproteinases-2 (TIMP2) is consistently downregulated in human HCC lesions; decreased TIMP2 is associated with liver invasion and poorer patient survival (Kai et al., 2016). Mechanistically, TIMP2 suppression is controlled by an HIF-1 α /miR-210/HIF-3 α feedback circuit in hypoxic HCC cells.

Role of HMGB1 in HCC

HMGB1 is a nuclear damage-associated molecule, induced upon hypoxia, that is associated with HCC invasion and metastasis. Meta-analysis showed a significant correlation between higher HMGB1 and poorer OS and progression-free survival in HCC (Wu et al., 2016). Yan et al. (2012) reported that hypoxia-induced extracellular release of HMGB1 can activate cysteinyl aspartate-specific proteinase-1 (caspase-1) via Toll-like receptor 4 (TLR4). Further, caspase-1 promoted interleukin (IL)-1 α /18 cleavage and release. Overexpression of HMGB1 or administration of recombinant HMGB1 enhanced

HCC cell invasion, whereas knockdown of HMGB1 inhibited invasion and pulmonary metastasis of HCC in mice. Recently, Chen et al. (2015) reported that HMGB1 promoted HCC progression by upregulating miR-21 and then subsequently suppressing MMP inhibitors [reversion-inducing cysteine-rich protein with kazal motifs (RECK) and TIMP3] in an IL6/STAT3-dependent manner. The results of these studies suggest that hypoxia promotes HCC invasion and metastasis by linking HMGB1 to the TLR4-mediated proinflammatory signaling pathway.

HMGB1 can also promote HCC growth by binding to mitochondrial DNA (mtDNA) and activating TLR9-mediated signaling (Liu et al., 2015). These authors showed that, in hypoxic HCC cells, nuclear HMGB1 was translocated to the cytoplasm and bound to free mtDNA, which further activated TLR9. The activation of HMGB1, mtDNA, and TLR9 depended on each other and was crucial for HCC cell proliferation in vitro and for HCC development in vivo.

Role of Beclin 1 in HCC

Beclin-1, a marker of autophagy, is altered in various cancers, including HCC. Qiu et al. (2014) reported that Beclin-1 expression was strong in 13/22 (59.1%), moderate in 15/53 (28.3%), and weak or negative in 7/28 (14.6%) HCC specimens. Compared with nontumor adjacent tissues ($n = 57$), Beclin-1 in HCC lesions ($n = 103$) was decreased significantly (Qiu et al., 2014). Reduced Beclin-1 correlated with cirrhosis, Edmondson grade, vascular invasion, microvessel density, and other molecular markers [such as PCNA (proliferating cell nuclear antigen), B-cell leukemia/lymphoma (Bcl)-2, and neuroepithelial cell transforming 1 (NET-1)] but was negatively correlated with BCL-2-associated X, apoptosis regulator (Bax) levels. Increased 5-year OS in HCC correlated to higher Beclin-1 levels with lower PCNA/Bcl-2/NET-1 or higher Bax. Thus, Beclin-1 could be regarded as an independent prognostic marker in HCC (Qiu et al., 2014).

Osman et al. (2015) investigated the role of Beclin-1 in HCC ($n = 65$). Beclin-1 was decreased in nearly half of HCC specimens (49.2%). Increased Beclin-1 was found mainly in cases with viral infection or higher HIF-1 α (64.6%). On the basis of HIF-1 α expression, the high or low HIF-1 α group was divided, representing either hypoxia or normoxia cases. In the high HIF-1 α group, increased Beclin-1 correlated well to many tumor parameters (i.e., grade, stage, size, multifoci). However, in the low-HIF-1 α group, an association of Beclin-1 and tumor parameters was not evident. These results support a conclusion that coexpression of Beclin-1 and HIF-1 α is associated with HCC progression.

Alteration of Glucose Metabolism in HCC

To adapt to an hypoxic microenvironment, most cancer cells undergo a mitochondrial-glycolytic metabolism shift. This is a hallmark of cancers, but the underlying molecular mechanism remains poorly understood. Sakamoto et al. (2015) reported that suppression of lysine-specific demethylase-1 (LSD1) downregulated HIF-1 α /glucose transporter 1 (GLUT1) and other glycolytic enzymes but upregulated a set of mitochondrial metabolism genes. LSD1 and GLUT1 were consistently co-overexpressed in human HCC tissues. These findings suggest that LSD1 is required for the glycolytic-mitochondrial

metabolism shift in HCC cells. Leung et al. (2015) reported that PIM1, a serine/threonine kinase, was overexpressed in 39% ($n = 56$) of human primary HCC cases. Hypoxia (1% O₂) significantly enhanced PIM1 expression in HCC cell lines. Knockdown of PIM1 reduced glucose uptake and suppressed HCC growth and metastasis in vivo. This evidence suggests that hypoxia-induced PIM1 is important for the mitochondrial-glycolytic metabolism shift in HCC. Jia et al. (2016) reported that miR-592 was downregulated in HCC specimens. These authors showed that overexpression of miR-592 reduced HIF-1 α , glycolytic metabolism, and HCC growth. In HCC cases, reduced miR-592 was associated with various malignant parameters or poorer OS.

Role of Neuroglobin in HCC

Hypoxia plays a fundamental role in carcinogenesis; however, the underlying mechanisms remain far from clear. An important reason might be the lack of an intracellular O₂ acceptor. The discovery of two intracellular members of the hemoglobin family, neuroglobin (Ngb) and cytoglobin (Cygb) in mammalian cells provides novel insights for investigating hypoxia-mediated responses in various diseases, including cancers (Qiu and Chen, 2014).

Neuroglobin, a monomeric heme-containing globin (17 kDa), exists predominantly in cells and tissues with a high metabolic rate, such as neurons/brain, endocrine gland cells, and liver (Qiu and Chen, 2014). The oxygen-binding affinity of NGB (1–2 torr) is similar to that of myoglobin and is much higher than that of hemoglobin (12 torr) (Qiu and Chen, 2014). However, the hexa-coordinated nature of the Fe²⁺ in the heme of NGB makes it difficult for the protein to release O₂ under physiologic conditions. It is generally hypothesized that Ngb may serve as an O₂/ROS sensor/carrier or NO/ROS scavenger (Ascenzi et al., 2014; Burmester and Hankeln, 2014; Qiu and Chen, 2014; Cai et al., 2016; Reuss et al., 2016).

Most previous studies reveal that Ngb can be upregulated in neural cells by acute hypoxia/ischemia, and that Ngb plays a protective role against ischemia (Sun et al., 2001; Chen et al., 2005) or oxidative stress (Ye et al., 2009; Antao et al., 2010). Recently, the expression of Ngb and its homologous Cygb were also investigated in various cancers. Emara et al. (2010, 2014) reported that Ngb/Cygb was widely expressed in many cancers through microarray analysis. However, Gorr et al. (2011) reported that Ngb, but not Cygb, was elevated in only a few types of tumors. The inconsistency of Ngb expression in cancers was also noted in other studies. For example, Shivapurkar et al. (2008) reported a decrease of Ngb, whereas Oleksiewicz et al. (2011) reported an elevation of Ngb, in lung cancers. This discrepancy might be the result of using different Ngb antibodies, or the differential expression of Ngb depending on specific cellular contexts (e.g., acute or chronic hypoxia, Ngb promoter hypermethylation). We had detected Ngb in liver, breast, lung, bladder, kidney, pancreas, and colon cancers and found a significant decrease of Ngb in HCC specimens only ($P < 0.0001$ versus adjacent nontumor tissues/normal liver tissues) (Zhang et al., 2013). Consistently, the level of Ngb mRNA was also decreased in HCC lesions. The downregulation of Ngb in HCC is in accordance with the alteration of Ngb level in neurons during chronic hypoxia (Hota et al., 2012; Liu et al., 2012b), suggesting that chronic hypoxia might be a major factor controlling Ngb expression in

HCC cells. In addition, hormones such as 17β -estradiol may also regulate Ngb expression in HCC (Fiocchetti et al., 2014).

Further studies revealed that Ngb suppressed HCC proliferation in vivo and in vitro (Zhang et al., 2013). These investigators found that Ngb overexpression significantly suppressed HepG2 cell proliferation and colony formation on soft agar and prevented the G0/G1-S transition, whereas knockdown of Ngb showed opposite effects. Moreover, Ngb overexpression suppressed the growth of HCC xenografts and reduced the tumor weight in nude mice. Mechanistically, Ngb bound to proto-oncogene serine/threonine-protein kinase (Raf)-1 and suppressed Raf-MEK-Erk in HCC cells. Interestingly, mutation of the oxygen-binding sites of Ngb (His-64) evidently altered its interaction with Raf-1 and the resulting Erk phosphorylation. Therefore, we propose that Ngb directly links the O_2 signal to the Raf-MEK-Erk pathway (Fig. 1). Considering that Raf is an important therapeutic target of sorafenib for HCC, targeting Ngb-Raf interactions might treat HCC in the future. In addition to Raf-1, Ngb also binds to other proteins such as phosphatase and tensin homolog (PTEN), AKT, G protein, cytochrome C, and 14-3-3 proteins, which are important signaling proteins controlling cell survival, death, and proliferation. It is conceivable that additional binding partners of Ngb will be identified. The binding affinity of Ngb to its client proteins is regulated by its O_2 -binding site, suggesting that Ngb directly links O_2 signals to intracellular signaling pathways. This function of Ngb as a signaling protein is distinct from that of HIF as a transcriptional factor, providing a novel point for investigating the effects and mechanisms of hypoxia in cancers.

Targeting Hypoxia for HCC Therapy

Role of YQ23. YQ23 is a synthetic tetrameric hemoglobin that facilitates oxygen delivery (Ling et al., 2014). YQ23 is taken up into cells by receptor(s)-mediated endocytosis (Man and Yau, 2015). Li et al. (2014) reported that the administration of YQ23 effectively decreased HCC incidence in an orthotopic rat HCC model. In addition, YQ23 reduced metastatic numbers and size in the lung following liver ischemia/reperfusion or hepatectomy (Li et al., 2014). Mechanistically, YQ23 inhibited HIF-1 α -dependent angiogenesis in combination with other anticancer drugs (Man and Yau, 2015). In addition, YQ23 suppressed the mobilization of endothelial progenitor cells via the C-X-C motif chemokine 10 (CXCL10)/CXCR3-tumor necrosis factor α /IL6 signaling pathway (Ling et al., 2014). The data support the possible usefulness of YQ23 for HCC therapy by its promotion of HCC cell oxygenation.

Role of Sorafenib

Sorafenib, a tyrosine kinase inhibitor, is a multitargeted agent and the only effective first-line drug for advanced HCC patients. However, drug-resistance can develop via hypoxia-mediated mechanisms (Llovet et al., 2008). Ma et al. (2014) showed that sorafenib upregulated HIF-2 α /VEGF/cyclin D1 but downregulated HIF-1 α . Knockdown of HIF-2 α enhanced the therapeutic results of sorafenib in HCC xenograft models (Liu et al., 2015). Chen et al. (2014) reported that sorafenib upregulated stromal cell-derived-1 (SDF1 α) and CXCR4 in hypoxic HCC cells and suppressed SDF1 α /CXCR4 signaling, resuming the anticancer effect of sorafenib despite the

presence of hypoxia. Further, suppression of the SDF1 α -CXCR4 signaling pathway significantly enhanced the therapeutic effects of sorafenib in orthotopic HCC tumors in mice upon hypoxia (Hato et al., 2014). Clinically, sorafenib in combination with or following transarterial chemoembolization (TACE) resulted in longer OS in HCC patients with portal vein invasion than did sorafenib monotherapy (Ha et al., 2016). Together, these results suggest that hypoxia significantly affects sorafenib therapy. Since hypoxia is heavily involved in sorafenib resistance in HCC therapy, it will be interesting to test whether the combination of sorafenib and YQ23 may have synergistic therapeutic effects for HCC. Theoretically, YQ23 cannot only promote tumor oxygenation but also suppress angiogenesis or inflammation via VEGF- or CXCR-signaling pathways in HCC.

Roles of Other Drugs

2-Methoxyestradiol (2-ME2) is a promising cancer therapeutic drug that downregulates HIF-1/2. Ma et al. (2014) reported that 2-ME2 effectively downregulated HIF-1 α /HIF-2 α , cyclin D1, VEGF, and lactate dehydrogenase A in HCC cells, which contributed to the resistance of the cells to hypoxia. Further, 2-ME2 in combination with sorafenib yielded better anticancer results in HCC by suppressing tumor angiogenesis. Celastrol is another promising anticancer drug that inhibits the hypoxia-induced HIF-1 pathway in HCC cells (Ma et al., 2014). In this study, celastrol prevented HCC growth by downregulating erythropoietin and VEGF, and suppressing mTOR, p70-S6 kinase 1, eIF4E, and Erk signaling.

Conclusion

Recent evidence supports the conclusion that hypoxia plays an important role in HCC development and therapy. The decrease of pO_2 within HCC lesions suggests that hypoxia plays a pivotal role during HCC development. Hypoxia marker HIF-1 α is a reliable indicator of poor prognosis for HCC patients. Hypoxia promotes HCC development via complicated mechanisms. Neuroglobin represents a novel type of hypoxia sensor in HCC. Improving HCC oxygenation or suppressing hypoxia-induced signaling are potential therapies for HCC.

Authorship Contribution

Wrote or contributed to the writing of the manuscript: Xiong, Qiu, Hu, Chen.

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